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Phosphorylation of the nucleocapsid protein of Hantaan virus by casein kinase II

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Hantaanvirus (HTNV) is the prototype of the genus Hantavirus, which belongs to the family *Bunyaviridae*. Hantaviruses are carried and transmitted by rodents and are known to cause two serious disease syndromes in humans i.e., hemorrhagic fever with renal syndrome (HFRS) and the hantavirus pulmonary syndrome (HPS). HTNV is an enveloped virus that contains a tripartite genome consisting of three negative-sense RNA segments (L, M, S), and the S and M segment of HTNV, respectively, encode the viral nucleocapsid protein (NP) and envelope glycoproteins. Possible phosphorylation motifs of casein kinase II (CKII) and protein kinase C (PKC) were identified in HTNV NP through bioinformatics searches. Sucrose gradient SDS-PAGE analysis indicated that dephosphorylated HTNV NP migrated faster than non-dephosphorylated NP, suggesting that HTNV NP is phosphorylated in infected Vero E6 cells. Immunoblot anaylsis of HTNV particles with anti-phosphoserine antibody and anti-phosphothreonine antibody after immunoprecipitation showed that viral particles are readily phosphorylated at threonine residues. *In vitro* kinase assay further showed that HTNV NP is phosphorylated by CK II, but not by PKC. Full length or truncated HTNV NPs expressed in *E. coli* were phosphorylated *in vitro* by CKII suggesting that phosphorylation may occur *in vivo* at multiple sites. Site specific mutagenesis studies suggest that HTNV NP phosphorylation might occur at unknown sites excluding the site-directly mutagenized locations. Taken together, HTNV NP can be phosphorylated mainly at threonine residues *in vivo* by CK II treatment.

***Keywords*:** Hantaan virus, nucleocapsid protein, phosphorylation, casein kinase II

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Introduction

The discovery of Hantaviruses, which belong to the family *Bunyaviridae*, may be traced back to 1951 to 1953, when United Nations troops were deployed during the border conflict between North and South Korea. More than 3,000 acute febrile cases were observed among the troops, one-third of which exhibited hemorrhagic manifestations. The overall mortality rate was 5–10% (Lee and Lee, 1977; Lee *et al*., 1978). The disease was initially termed Korean hemorrhagic fever (KHF) but is now referred to as hemorrhagic fever with renal syndrome (HFRS). Despite considerable efforts, it took about 25 years until Lee and Lee identified the field mouse, *Apodermus agrarius*, as the rodent reservoir of the virus (Lee and Lee, 1976). This discovery led to the eventual isolation of the virus (Lee and Lee, 1977; Lee *et al*., 1978). The virus was named Hantaanvirus (HTNV), after the Hantaan River in a northern area of South Korea close to the location of early Korean HFRS outbreak cases, and became the prototype of the Hantavirus genus (Lee *et al*., 1978).

Viruses of the genus Hantavirus have three segmented negative sense RNAs as their genome. The nucleocapsid protein (NP), encoded by the S segment, of HTNV contains 429–433 amino acid residues and has a/ molecular mass of approximately 50 kDa. This NP binds and forms ribonucleoproteins and encapsidates the genomic RNAs into the three viral segments (Alfadhli *et al*., 2001; Mir and Panganiban, 2004). It is the most abundant viral protein in infected cells, where it localizes to the cytoplasm and forms inclusion bodies and filamentous structures (Elliott and Schmaljohn, 2013). It is multifunctional, involved in various interactions with cellular components during the HTNV life cycle. In addition, the NP has important functions in viral RNA replication and assembly, as well as protection of the RNA genome (Elliott and Schmaljohn, 2013). Previous studies have demonstrated that when hantaviruses infect cells, the NP interacts with a number of cellular proteins. For instance, the NP of Puumala virus interacted with Daxx (Li *et al*., 2002). The NPs of HTNV and Tula virus interact with ubiquitin-conjugating (Ubc) enzyme and small ubiquitin-like modifier 1 (SUMO-1) (Kaukinen *et al*., 2003).

Although HTNV NP plays important roles in the interaction with various cellular proteins during the life cycle of the virus, the phosphorylation status of HTNV NP is not yet characterized. We firstly reported in this study that HTNV NP could be phosphorylated *in vitro* by casein kinase II (CKII).

Table 1. Possible HTNV NP phosphorylation sites

Net Phos 2.0		Kinase Phos 2.0		
Serine Locations (AA)	Threonine Locations (AA)	Serine Locations (AA)	Threonine Locations (AA)	Mutagenized locations (AA)*
163	**45**	**163**	71	**45** (Threonine)
164	71	**164**	116	**163** (Serine)
187	116	180	148	**164** (Serine)
217	127	221	149	-
221	142	233	200	-
301	148	**294**	261	**294** (Serine)
351	149	301	341	-
359	194	345	347	-
360	261	371	350	-
412	341	412	369	-
422	350	**422**	408	**422** (Serine)

429 HTNV NP amino acid sequences are searched by Net Phos 2.0 and Kinase Phos 2.0 databases to identify possible NP phosphorylation sites of Serine or Theronine residues. *Site-directed mutation sites are selected to abolish Serine or Theronine residues.

Materials and Methods

Cell line and virus

Vero E6 cells was purchased from the American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 2% or 10% fetal calf serum and 50 U/ml penicillin and streptomycin in a 5% CO_2 humidified atmosphere. HTNV 76-118 strain was supplied by Dr. KeumYong Kim (School of Medicine, The Catholic University of Korea, Seoul, Korea).

Isolation of HTNV full length S genome and generation of truncated S fragments.

Isolation strategy for full length of HTNV S genome was adopted from Schmaljohn *et al.* (1986) work. Briefly, full length of the S fragment was amplified by reverse transcription PCR. The PCR products were then cloned into pGEM T easy vector (Promega) and all fragments were confirmed by sequencing. Primers used for amplification of full length and truncated S fragments were as follow.

NP full length/T1 forward:
5′-GAGCTCATGGCAACTATG GAGGAATTA-3′
T1 reverse:
5′-GTCGACTTACCAGTCTGCTGTCTGTCC-3′
T2 forward:
5′-GAGCTCCTGAGCATCATCGTCTATCTT-3′
T2 reverse:
5′-GTCGACTTATGTTCTATATCTACCAGGTG-3′
T3 forward:
5′-GAGCTCGCAGTCTGTGGGCTCTACCC-3′
T3 reverse:
5′-GTCGACTTATGTTGGTGGACAACGGTC-3′
T4 forward:
5′-GAGCTCTGTTTGTTTATAGCAGGTATTGC-3′
NP full length/T4 reverse:
5′-GTGTCGACTTAGAGTTTCAAAGGCTCTTG-3′

HTNV particle dephosphorylation assay

Vero E6 cells were infected with the HTNV 76-118 strain. After 7 days of incubation, the infected cells were destroyed for release of virus by repeated freezing and thawing cycles in dry-ice ethanol bath. Dephosphorylation assays of virus were carried out using CIP assay kit (Calf intestine alkaline phosphatase, Promega). 20 U or 50 U of CIP with buffer were added to virus emulsion for incubation at 37°C for 30 min. Dephosphorylation status was determined by running of 10–15% gradient SDS-PAGE gel then immunoblotting with HTNV specific antibody.

Site directed mutagenesis of HTNV S genome and bacterial expression

The Serine or Threonine residues of truncated HTNV S fragments (See Fig. 1A for location of each residue) were site-directly mutated to alanine residue by site-directed mutagenesis kit (Promega). T1, T2, and T4 point mutations were confirmed by sequencing. However, T3 point mutation was not generated. Total and truncated versions of S genome were cloned into pGEX 4T-1 (Amersham Pharmacia) harboring GST fusion system and pQE-30 (Qiagen) expression vectors harboring 4x Histidine tagging system respectively and then bacterially expressed in the *E. coli* JM109 strain. Expressed proteins were then purified by His-tagged (Promega) or GST-tagged protein purification system (Clontech) according to the manufacturer's instructions.

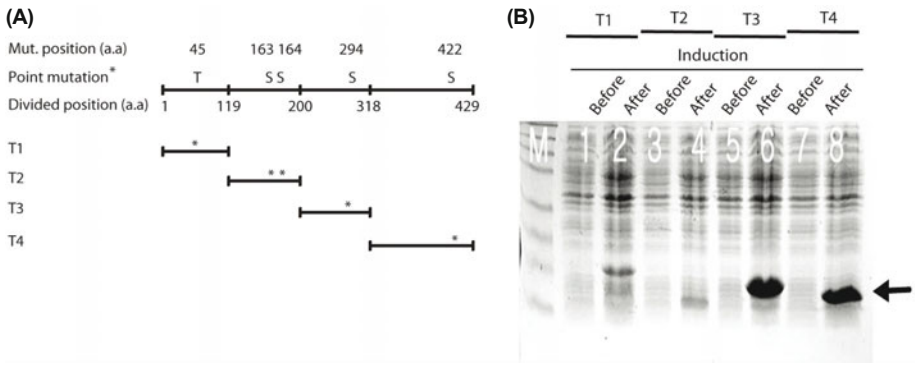

Fig. 1. Possible HTNV NP phosphorylation sites and generation of full length or truncated proteins (including point mutants). (A) In order to identify possible NP phosphorylation sites, ExPASY Scan Prosites, MEME System, MOTIF, Pattern Find Server, Profile Scan Server were used. At least seven possible phosphorylation residues are searched and indicated as " * ". The NP genome was also divided into four regions as described in the Results. * indicates site-directly mutagenized locations to abolish serine or theronine residues. Each point mutation is verified by sequencing. (B) The truncated versions of the NP were expressed in *E. coli* and each protein expression was verified in SDS-PAGE gel by comparing prior to and following induction.

HTNV particle phosphorylation assay

Vero E6 cells were infected with the HTNV 76-118 strain. After 7 days of incubation, the infected cells were destroyed for release of virus by repeated freezing and thawing cycles in lysis buffer containing protease inhibitors. After spinning down to discard cell debris, the soluble fractions were reacted with anti-HTNV antibody for 2 h at 4°C, and precipitates were then recovered by incubation with protein G agarose beads (Pierce) for overnight at 4°C. After through washing, proteins bound to the agarose beads were separated by SDS-polyacrylamide gel electrophoresis and were analyzed by immunoblotting with specific antibodies, biotin-conjugated anti-phosphoserine monoclonal antibody and biotin-conjugated anti-phosphothreonine monoclonal antibody (Sigma).

In vitro kinase assay

For CKII kinase assay, purified proteins were incubated with 100 μM cold ATP (Promega), 10uCi Adenosine 5′-[γ-32P] triphosphate (>185TBq/mmol,>5,000 Ci/mmol, Amersham Pharmacia Biotech), 20 or 50 U CKII (Promega) and CKII reaction buffer (25 mM Tris-Cl, pH 7.4, 0.2 M NaCl, 10 mM $MgCl_2$) at 37°C for 45 min. β-Casein (Sigma) is used for positive control in CKII kinase assay. For PKC kinase assay, purified proteins were incubated with 100 μM cold ATP (Promega), 10 uCi Adenosine 5′-[γ-32P] triphosphate ±0.6 mg/ml phosphatidyl serine (Sigma), 1 μl of PKC (Promega) and PKC reaction buffer (20 mM HEPES, pH 7.4, 1.67 mM $CaCl_2$, 1 mM DTT, 10 mM $MgCl_2$) at 30°C for 45 min. Neurogranin (28-43) (PKC) peptide substrate (Promega) is used for positive control in PKC kinase assay. Kinase reactions were terminated by addition of an equal volume of 2 × SDS sample buffer. The samples were boiled for 5 min prior to running the 12% SDS-polyacrylamide gel electrophoresis (PAGE). The gel was then fixed, dried, and autoradiographed.

Results

Possible HTNV NP phosphorylation sites and generation of full length / truncated proteins including point mutants

The S segment of HTNV contains 1,696 nucleotides that code for nucleocapsid protein (NP) of 429 amino acids (Schmaljohn *et al.*, 1986). In order to identify possible NP phosphorylation sites, Netphos 2.0 and Kinase Phos 2.0 databases were used. As shown in Table 1, eleven possible phosphorylation sites of Serine or Threonine residues are searched and five possible sites are selected for site-directed mutagenesis analysis. The NP genome was divided into four regions (1, 2, 3, and 4). Region 1 (amino acids 1–118), region 2

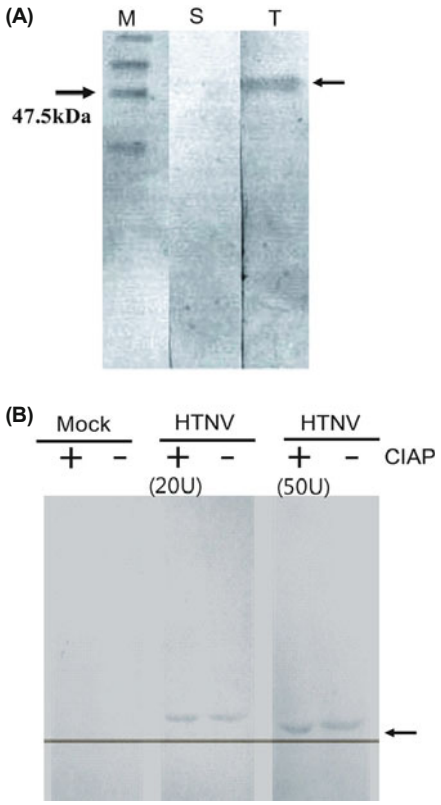

Fig. 2. HTNV particles are phosphorylated and NPs (nucleocapsid proteins) can be dephosphorylated by a phosphatase. (A) Purified HTNV particles were blotted with anti-phosphoserine (S) or anti-phosphothreonine (T) antibodies. Viral particles (About 47.5 kDa) are richly phosphorylated in threonine residues. M, size marker. (B) HTNV NPs were dephoshorylated upon 50 U dose of CIAP (calf intestine alkaline phosphatase) treatment. 50 U CIAP treatment caused a NP bandshift due to dephosphorylation event (arrow). Mock, mock infected.

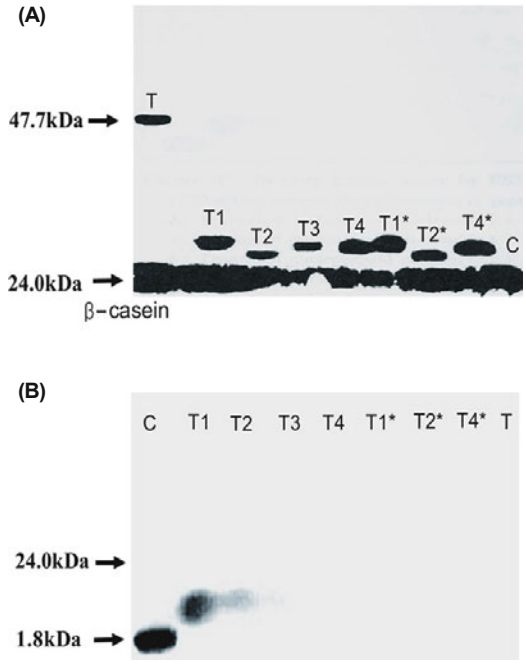

Fig. 3. HTNV NP (nucleocapsid protein) can be phosphorylated by CKII (casein kinase II). Full length or truncated NPs were challenged with CKII for *in vitro* kinase reaction as described in the 'Materials and Methods'. All the proteins were phoshorylated by CKII. C, positive control only (β-casein). (B) Total or truncated NPs were challenged with PKC for in vitro kinase reaction as described in the Materials and Methods. All the proteins were not phoshorylated by PKC. The signals of T1 and T2 lane are not relevant based on size marker. C, positive control (neurogranin peptide).

(amino acids 119–199), region 3 (amino acids 200–317) and region 4 (amino acids 319–429) were mapped on NP as shown in Fig. 1A. Next, truncated and point-mutated proteins were constructed according to the NP map to determine the location of NP phosphorylation residues. In addition, site-directed mutagenesis of five NP phosphorylation candidate sites was conducted to abolish serine or threonine residues (Fig. 1A and Table 1). The full length NP genome and truncated versions of the NP genome were expressed in *E. coli* (Fig. 1B) and purified by GST or histidine tags as described in 'Materials and Methods'.

HTNV particle phosphorylation and dephosphorylation

Vero E6 cells were infected by HTNV. At 7 days post-infection, viral particles were released from the infected cells by repeated freezing and thawing. Virus particles were then isolated using the protein G purification method and immunoblotted using anti-phosphoserine or anti-phosphothreonine antibodies as described in 'Materials and Methods'. As shown in Fig. 2A. HTNV particles, which are about 47.5 kDa, were readily detected by phoshotheronine antiserum indicating that HTNV particles are heavily phosphorylated in threonine residues. In order to examine whether viral particles can be dephosphorylated by a phosphatase treatment, these purified viral particles were treated with calf intestine alkaline phosphatase (CIAP) prior to gradient SDS-PAGE analysis as described in Materials and Methods. In this gradient SDS-PAGE gel, treatment of 50 U of CIAP induced a viral particle band shift showing that viral particles can be dephosphorylated by CIAP as shown in Fig. 2B. Taken together, HTNV particles are phosphorylated in Vero E6 infected cells and can be dephosphorylated by CIAP treatment.

HTNV NP (nucleocapsid protein) phosphorylation by CKII (casein kinase II)

Full length or truncated versions of HTNV NPs were challenged with CKII for *in vitro* kinase reaction as described in 'Materials and Methods'. As shown in Fig. 3A, full length or truncated versions of NPs were all phosphorylated by CKII. The site-directly mutated proteins were also phosphorylated by CKII showing that CKII can be a candidate cellular kinase for NP phosphorylation in Vero E6 infected cells as shown in Fig. 3A. Because T1, T2, and T4 regions were all phosphorylated, it is suggesting that the mutagenized serine or threonine locations are not the phosphorylation sites. In contrast, when the full length or truncated NP were challenged with PKC for *in vitro* kinase reaction (Fig. 3B), none of these proteins were phosphorylated suggesting that PKC is not the cellular kinase for NP phosphorylation. Taken together, HTNV NP can be readily phosphorylated in a regional independent manner by CKII by *in vitro* kinase treatment.

Discussion

Hantavirus NPs are about 420-430 residues and approximately 50-kDa proteins, the N-terminal 75 residues of which carry two coiled coil motifs that facilitate trimerization and nucleocapsid protein trimers that are believed to be Hantavirus particle assembly intermediates (Alfadhli *et al.*, 2001, 2002). Hantavirus NPs are relatively conserved among hantaviruses and is highly immunogenic in laboratory animals and humans; it has been shown to induce efficient protective immunity in animal models (de Carvalho *et al.*, 2002). In murine models, HTNV infection elicited a strong nucleocapsid protein–specific CD8(+) T cell response 8 days after infection (Woo *et al.*, 2005). Importantly, Taylor *et al.* (2009) reported that HTNV NP binds to importin alpha proteins and inhibits tumor necrosis factor alpha-induced activation of nuclear factor kappa B (NF-κB). Collectively, HTNV NP appears to be emerging as a multifunctional protein with roles in several virus-host cell interactions in addition to viral replication. In the case of rabies virus, the nucleoprotein N is phosphorylated by CKII and the N protein is known to play an important role in the process of viral transcription and replication (Wu *et al.*, 2003). Thus, it is likely that CKII-mediated phosphorylation of viral nucleoproteins, including HTNV NP, could play an important role in viral transcription and replication in general.

Phosphorylation and dephosphorylation of cellular or viral proteins plays an important role in regulating protein functions during virus/host interactions. There are two major kinases that phosphorylate tyrosine residue or ser/theronine residue in host cells. It is thought that phosphorylation of paramyxovirus proteins must be carried out by host Serine/theronine kinases owing to lack of known viral kinases (Lenard, 1999). At present, two host Serine/theronine kinases, CKII and PKC, have been proposed as the main host kinases that phosphorylate paramyxovirus P proteins. CKII is thought to phosphorylate the P proteins of respiratory syncytial virus (Mazumder *et al.*, 1994) and measles virus (Das *et al.*, 1995) and N protein of rabies virus (Wu *et al.*, 2003). While the role of CKII in phosphorylation of the P proteins has been studied most, interestingly, owing to the nature of CKII (ubiquitous expression and multiple subunits and isoforms), it has never been shown that CKII is directly involved in paramyxovirus replication in infected cells. Recently, Sun *et al.* found that AKT plays a critical role in replication of many paramyxoviruses by studying a prototypical paramyxovirus, PIV5 (Sun *et al.*, 2008). They reported that AKT, a ser/theronine kinase, also known as PKB, interacts with P protein as well as phosphorylates P protein *in vitro*. Both AKT inhibitors and siRNA targeting AKT1 reduced parainfluenza virus 5 replication. Furthermore, inhibitors of AKT also reduced the replication of several other paramyxoviruses including respiratory syncytial virus (RSV), indicating that AKT plays a critical role in paramyxovirus replication. At present, AKT binding sites and phosphorylation target sites within paramyxovirus P proteins have not been identified.

Although HTNV NP plays an important role in interacting with various cellular proteins during the life cycle of the virus, phosphorylation status of HTNV NP has not yet characterized. We firstly reported in this study that HTNV NP can be phosphporylated by casein kinase II (CKII). Although the five candidate Serine/theronine (S/T) mutants were not responsible for HTNV NP phosphorylation in this experiment, there are many untested candidate S/T phosphorylation sites as shown in Table 1. Since the Theronine residues are mainly phoshporylated in HTNV virion particle analysis,

HTNV NP amino acid positions of 71, 116, 341, and other Threonine residues could be possible NP phosphorylation sites by CKII (Table 1). Further studies of HTNV NP phosphorylation such as *in vivo* identification of host cellular kinases and the exact location of phosphorylation residues, may provide important clues of the molecular basis of HTNV pathogenesis and drug development.

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Disclosure

The authors has no conflicts of interest to disclose.

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